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Tyramide Signal Amplification (TSA)-FISH Applied to Mapping PCR-Labeled Probes Less than 1 kb in Size

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BioTechniques 27:608-613 (September 1999)

ABSTRACT

Tyramide signal amplification (TSA)-FISH was used to map one mouse and two human DNA probes of less than 1 kb in size. The two human probes were 319 and 608 bp, and the mouse probe was 855 bp. Probes, made from PCR products, were labeled by incorporating biotin-11-dUTP (human) and biotin-16-dUTP (mouse) during PCR amplification. Signals were readily observed in both interphase and metaphase cells following TSA-FISH for all three genes, whereas conventional FISH experiments produced no signals. The two human ATP-binding cassette (ABC) genes, EST883227 (GenBank® Accession No. AA243820) and EST990006 (GenBank Accession No. AA348546), mapped to human chromosomes 7p21 and 17q25. The mouse gene, c-myc (exon 2) mapped to band D2 of mouse chromosome 15. These findings demonstrate the ability of this technique to map small probes (PCR products and expressed sequence tags) of less than 1 kb through highly increased signal amplification.

INTRODUCTION

Fluorescence in situ hybridization (FISH) is a technique routinely used for chromosome mapping in humans, mouse and other mammals through visualization of a probe hybridized to metaphase chromosomes and interphase nuclei (2). Conventional FISH involves the labeling of DNA with a reporter molecule (e.g., biotin) by polymerase chain reaction (PCR) or nick translation (16), followed by hybridization of probe and target DNA and incubation with immunofluorescent reagents [e.g., avidin-fluorescein isothiocyanate (FITC)] (16). One limitation of FISH has been the size of the fragment that could be detected once labeled. Conventional FISH has not proven reliable enough to map single copy probes that are smaller than 1 kb in size.

In this study, we explored the sensitivity of an alternative FISH method, Tyramide Signal Amplification (TSA)-FISH (NEN Life Sciences Products, Boston, MA, USA; **http://www. nenlifesci.com**), which has been used in a number of other applications, such as mRNA in situ hybridization (ISH) on formaldehyde-fixed and paraffin-embedded tissues (15), co-localization of HuC and HuR genes (members of Eval family of RNA-binding proteins) on the same chromosomal preparation (metaphase spreads) using small probes (1.2 and 2.5 kb, respectively) labeled by nick translation (19) and detection of the human cytomegalovirus immediate early (HCMV-IE) gene integration site (17).

TSA-FISH is a multi-step procedure involving in situ hybridization with a labeled probe, detection of a hybridized target with streptavidin-horseradish peroxidase (SA-HRP), signal amplification (incubated with tyramide-biotin), detection of amplified signal [incubation with streptavidin-fluorescein (SA-FITC)] and imaging (10). Amplification is increased greatly in this procedure by the biotin-labeled tyramide, a phenolic compound, that reacts and binds with electron rich moieties (e.g., HRP) that catalyzes the binding of the tyramide to the surface of the cell preparations on the slide. Therefore, tyramide-based detection always requires a peroxidase-conjugated antibody. The key to TSA-FISH is the multiple deposition of the reactive biotinylated-tyramide, resulting in the amplification of a fluorescent signal up to 1000-fold (4,9–11,13,14,18).

The two human probes used in this study EST990006 (GenBank® Accession No. AA348546) and EST883227 (GenBank Accession No. AA243820) belong to the ATP-

binding cassette (ABC) superfamily. These genes are members of a large family of proteins that code primarily for transport proteins involved in the movement of important molecules across cell membranes including lipids, peptides, hydrophobic and chemotherapeutic drugs. ABC genes are involved in a number of human genetic diseases including X-linked adrenoleukodystrophy, cystic fibrosis and Stargardt disease (Reference 1 and **http://www. gene.ucl.ac.uk/users /hester/abc.html**).

C-myc is an proto-oncogene and is a member of the helix-loop-helix/leucine zipper family of transcription factors (6,7). When *c-myc* is activated by mutations, it acts as a cell proliferation regulator in mammalian cells (9). *C-myc* over-expression has been found in many human and murine neoplasias, as for example in plasmacytomas, and was previously localized by FISH in the mouse to chromosome 15, band D2 (3).

As a control, we attempted to map the small, labeled probes by conventional FISH before mapping with TSA-FISH. However, no signals were visible using conventional FISH. Therefore, we proceeded to map the probes by using TSA-FISH with minor modifications in the protocol. This study is the first application of TSA-FISH for mapping of small probes (less than 1 kb in size) labeled by PCR in metaphase chromosome spreads.

MATERIALS AND METHODS

PCR Conditions

The human DNA probes were labeled with biotin-11-dUTP, and the mouse DNA probe was labeled with biotin-16-dUTP using PCR amplification. The human probe was synthesized by PCR from human genomic DNA by the following procedure. For each reaction, the master mixture included 10× buffer containing 15 mM $MgCl_2$ (PE Biosystems, Foster City, CA, USA), 2 mM dNTP, 5 U/µL *Taq* DNA Polymerase (PE Biosystems), forward and reverse primers (1 µM each) and double-distilled (dd)H_20 up to 24 µL. For the dNTP, the final concentrations were: 0.2 mM each dATP, dCTP and dGTP, 0.15 mM dTTP and 0.03 mM biotin-11-dUTP. The final reaction mixture contained 1 µL of DNA (20–40 ng) and 24 µL of the master mixture. PCRs were incubated at 95°C for 2 min followed by 12 cycles: denature at 94°C for 12 s, anneal at 65°C for 20 s and extend at 72°C for 55 s. The reactions were incubated next for 30 cycles at 94°C for 12 s, 50°C for 20 s and 72°C for 55 s. After completion of the last cycle, the reaction was extended at 72°C for 10 min and held at 4°C. Two repeats of each PCR were pooled, and the OD_{260} read. Primers for EST883227, forward 5′-CCATAGCAGTTATTG-CATTG-3′ and reverse 5′-GATCATATAACCTCTGCAG-AAG-3′, and EST990006, forward 5′-CGGTTCCATCCAA-CACCTG-3′ and reverse 5′-CAATGGAACCAGTATGGC-CTG-3′, generated 319- and 608-bp fragments, respectively.

Mouse probe DNA was synthesized by PCR according to the method previously described (8), with the exception that 25% of the dTTP was substituted with biotin-16-dUTP. The following primer pairs, forward primer, 5′-TCCTTC-CTCTCGTTTCCCCGCCCCCCTCTGCTTT-3′, and reverse primer, 5′-TGGAACTGTGTGGAGGTGTATGGGGTGTA-G-3′, annealed in the exon 2 region and generated a 855-bp fragment for the *c-myc* locus in mouse.

Fluorescence In Situ Hybridization

Two experiments were run concurrently to measure the sensitivity of TSA-FISH. Mapping of the probes was attempted with both conventional FISH (12) and TSA-FISH. In each experiment, the mapping of probes involved looking for the presence of signals in twenty normal metaphase spreads. For the human probes, metaphase spreads were used from peripheral blood lymphocytes. For both conventional and TSA-FISH, we followed the same protocols up to and including the blocking step, detailed below. Two different amounts of the DNA from the human probes were tested, 0.5 and 3.0 µL (1 µL = 600 ng), by conventional FISH and TSA-FISH.

Slide Pretreatment

Slides were pretreated with 3% hydrogen peroxide (H_2O_2) in methanol (10 min) and rinsed in 1× phosphate-buffered saline (PBS), to minimize background and quench endogenous peroxidase activity. For removal of excess cytoplasm, the slides were then pretreated by using the digestive enzyme, pepsin (Sigma, St. Louis, MO, USA). For pepsin pretreatment, 10 µL of pepsin from a 10% stock solution (100 mg/mL) were dissolved in sterile water placed into 100 mL of pre-warmed (37°C) 0.01 M HCl, pH 2.0–2.5. The slides were placed into a coplin jar containing the pepsin/HCl solution, for 5 min at room temperature. Following pretreatment, slides were fixed with 1% formaldehyde in 1× PBS/$MgCl_2$ and washed once with 1× PBS (at room temperature). Slides were then dehydrated in an ethanol series, 70%, 90% and 100% for 3 min each (11).

DNA Precipitation, Denaturation and Hybridization

Human probes were prepared by combining 0.5 or 3 µL of probe (DNA concentration 600 ng/µL), 10 µL of Human Cot-1® DNA (500 µg, 1 mg/mL; Life Technologies, Gaithersburg, MD, USA) and 1 µL (10 µg) salmon sperm DNA (10 mg/mL; Sigma). The mouse probe consisted of 25 µL of probe (DNA concentration, 10 ng/µL) and 2 µL salmon sperm DNA.

The probes were precipitated by adding 1/10 volume Na-acetate (3 M) and 2.5–3.0× total volume of cold 100% ethanol, vortex mixed and stored at -20°C overnight or at -80°C for 1 h. Next, the probes were centrifuged at 14 000 rpm at 4°C for 30 min in a Model 5417 R Eppendorf® Microcentrifuge (Brinkmann Instruments, Westbury, NY, USA). The supernatant was removed, and the tubes were placed in a speed-vac for 10 min (at medium heat) to dry the pellet. Then 5 µL of deionized formamide (pH 7.0–7.5) were added to each probe, the tubes were vortex mixed and incubated at 37°C for 30 min (shaking). Five microliters of the master mixture [20% dextran sulfate in 2× standard saline citrate (SSC), pH 7.0] were added to the probe mixture, the tubes were vortex mixed and spun down. The probe DNA was denatured at 85°C for 5 min in a water bath. The slides were denatured by applying 120 µL of 70% formamide in 2× SSC (pH 7.5), covered with a 24 × 60 mm^2 coverslip and heated at 80°C on a slide warmer for 1.5 min (time may vary depending on slides). The slides were immediately dipped in freshly prepared ice cold 70% ethanol for 3 min, followed by 90% and 100% ethanol dips of 3 min each. The denatured probe DNA was applied to the dry, denatured slides and covered with an

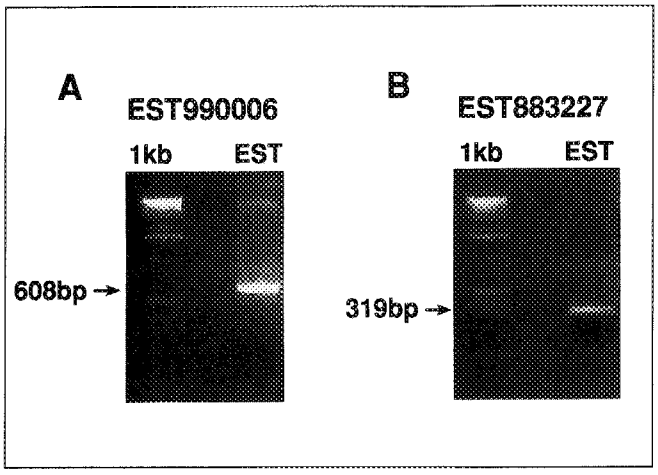

Figure 1. Human probes for EST883227 and EST990006. This figure displays the results of PCR amplification of the two human ABC genes (A and B). The PCR products were run on 2% agarose gels using ethidium bromide staining and a 1-kb ladder as the marker.

18-mm^2 coverslip, sealed with rubber cement and placed in a light-proof/hybridization chamber for 24–48 h at 37°C.

Detection

Conventional FISH. Slides were: *(i)* washed three times in 50% formamide/2× SSC (pH 7.0–7.5) (45°C for 5 min each); *(ii)* washed three times in 0.1 × SSC (60°C for 5 min each); *(iii)* dipped in 4× SSC/Tween® 20 buffer at 45°C; and *(iv)* incubated in blocking solution [3% bovine serum albumin (BSA) in 4× SSC/Tween 20] for 30 min at 37°C using 120 µL per slide.

Following blocking, the conventional FISH slides were treated with avidin-FITC antibody (dilution 1:200) for 1 h at 37°C, washed 3 times with 4× SSC/Tween 20, stained with 4′6-diamidine-2-phenylindole (DAPI) (for 5 min) and washed in water (for 5 min), covered with antifade reagent (paraphenylenediamine antifade; Sigma).

TSA-FISH. TSA™ Indirect *in situ* Hybridization Kits were obtained from NEN Life Sciences Products (biotinyl

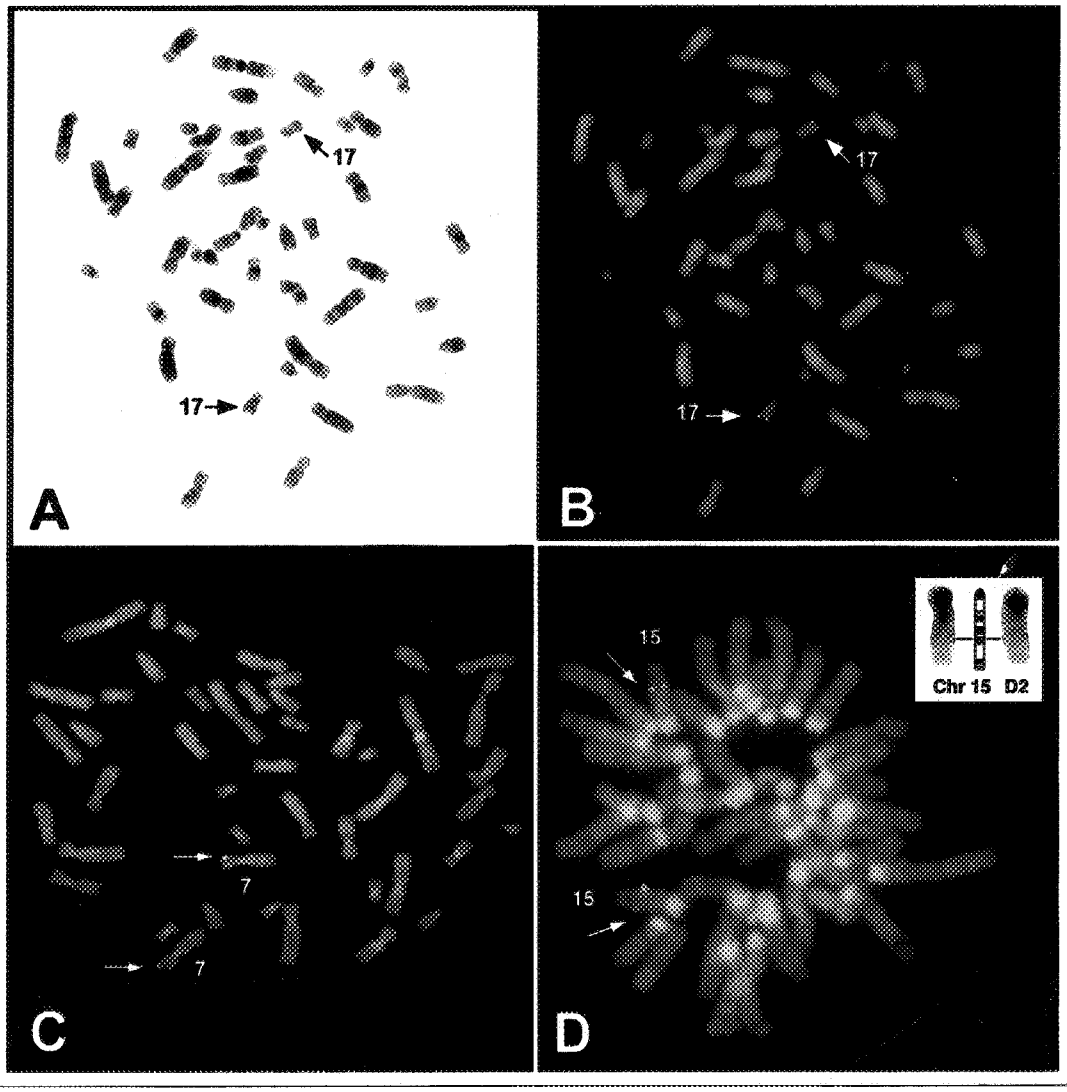

Figure 2. TSA-FISH detection of two human ABC genes and the *c-myc* mouse (exon 2) gene. (A) An inverted-DAPI metaphase spread of a normal human lymphocyte cell. (B) The identical metaphase spread with the chromosomes stained with DAPI (blue). The two green signals at the telomere regions of chromosome 17q25 (arrows) are the result of hybridization with the human ABC gene EST990006 (608 bp). (C) The results of hybridizing the ABC gene EST883227. This probe was 319 bp in size. (D) Mouse metaphase chromosomes with the results of the hybridization of mouse *c-myc*, exon 2 gene (855 bp). The resulting signals, shown in green pseudocolors (arrows), confirm the assignment of this gene to mouse chromosome 15, band D2 (see inset).

tyramide; Catalog No. NEL 730 for 200–600 slides and NEL 730A for 50–150 slides). TSA-FISH detection followed the protocols as described by the manufacturer with minor modifications. Higher stringency washes (0.1× SSC) were used to reduce background, and the TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) was brought to pH 7.0–7.5.

The biotin label was detected with SA-HRP (supplied in the kit) 1:100 in TNB [0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Blocking Reagent (supplied in kit)] (120 µL per slide) for 30 min at room temperature and washed 3 times for 5 min each in TNT buffer (room temperature), while shaking.

For the tyramide amplification procedure, three different methods of detection were tested: *(i)* direct detection using tyramide-Cy3 (Tyr-Cy3); *(ii)* direct detection using tyramide-fluorescein (Tyr-fluorescein); and *(iii)* indirect detection using biotin-avidin-FITC (tyramide bound to biotin; Tyr-Bio).

To each slide, 300 µL of each tyramide conjugate were applied at various dilutions (Tyr-Bio 1:50; Tyr-Cy3 1:500; Tyr-fluorescein 1:250) without use of a coverslip, for 5 min at room temperature. The tyramide solution was removed, and the slides were washed 3 times for 5 min each with TNT at room temperature. Fluorochrome-conjugated avidin (Av-FITC) diluted 1:200 in TNB was used to detect Tyr-Bio. Slides were incubated for 30–45 min at 37°C. Slides were then washed with TNT buffer 3 times for 5 min each at room temperature, counterstained with DAPI (80 ng/mL in 2× SSC) for 5 min at room temperature, and rinsed in water for 5 min, while shaking. The slides were dehydrated sequentially in 70%, 90% and 100% ethanol. To each slide, the antifade was applied and covered with a coverslip (24 × 60 mm^2) before image acquisition.

Image Acquisition

Fluorescent signals were imaged separately with the appropriate filter set using a Zeiss Axioskop® (Carl Zeiss, Thornwood, NY, USA) or Leica Epifluorescence Microscope (Leica Imaging Systems, Cambridge, England, UK) equipped with a cooled Model CH250 charge-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA). The filters used with a Leica DMRBE Microscope (Leica Imaging Systems) were TR1 (DAPI), TR2 (FITC) and TR3 [tetramethylrhodamine isothiocyanate (TRITC)] (Chroma Technology, Brattleboro, VT, USA). The DAPI and FITC images were merged and pseudocolored using Image™ (Oncor, Gaithersburg, MD, USA) or Q-FISH® (Leica Imaging Systems) software as described previously (11).

All of our images were initially imaged with the Q-FISH software, which reduces background automatically when images are merged, but does not enhance the images otherwise when they are captured (Figure 3). In addition, we find with TSA-FISH, the signals are brighter than those visualized with conventional FISH, and therefore require reduced exposure times so as to not saturate the images of the signals.

RESULTS

We explored the limits of sensitivity of TSA-FISH for gene mapping of probes of less than 1 kb in size. Twenty metaphase spreads from normal human lymphocyte and/or mouse spleen preparations were analyzed for TSA-FISH and routine FISH. TSA-FISH resulted in bright, amplified signals, whereas conventional FISH methods gave no detectable signals from the human and mouse probes. The indirect method (Tyr-Bio) using avidin-FITC provided the best results, i.e., clear, distinct signals on one or both of the homologues; whereas both the Tyr-Cy3 and Tyr-fluorescein resulted in high background. For EST990006, we observed two signals (doublets) on both homologues of chromosome 17, in the long arm, on all twenty metaphases. For EST883227, the 319-bp probe, we obtained the following data from the 20 metaphase spreads examined: four cells had no signals; four had one signal (on one homologue of chromosome 7 in the short arm); eight had one signal on both homologues of 7p (Figure 2C); four had 3 signals (2 on one homologue and one on the other homologue of 7p); and 0 metaphase spreads were seen with 4 signals or symmetrical doublets on both homologues of chromosome 7.

Using TSA-FISH, we successfully mapped EST990006, EST883227 and the *c-myc* gene using small PCR indirect labeled (biotin) DNA probes [608 and 319 bp (Figure 1) and 855 bp, respectively]. EST990006 mapped to human 17q25, EST883227 mapped to human 7p21 and *c-myc*, as expected, mapped to band D2 on mouse chromosome 15 (Figure 2). The cDNA of these two human ABC genes had been previously sequenced and determined to be unique through Basic Local Alignment Search Tool (BLAST) searches of the expressed sequence tag (EST) and nonredundant databases (**http://www.ncbi.nlm.nih.gov/BLAST/**). TSA-FISH mapping was undertaken after repeated attempts to map the first gene, EST990006, using the GeneBridge 4 Radiation Hybrid Panel (Research Genetics, Huntsville, AL, USA). The second gene, EST883227 was previously mapped using the GeneBridge 4 Radiation Hybrid Panel to 7p15-p21.

Human EST990006 is a contig containing the human est H65920 (Genbank Accession No.) and is in the unigene cluster Hs.38095. Cluster Hs.38095 has been mapped by the Sanger Centre (Cambridge, England, UK) to human chromosome 17 (D17S794-D17S795; **http://www.ncbi.nlm.nih.**

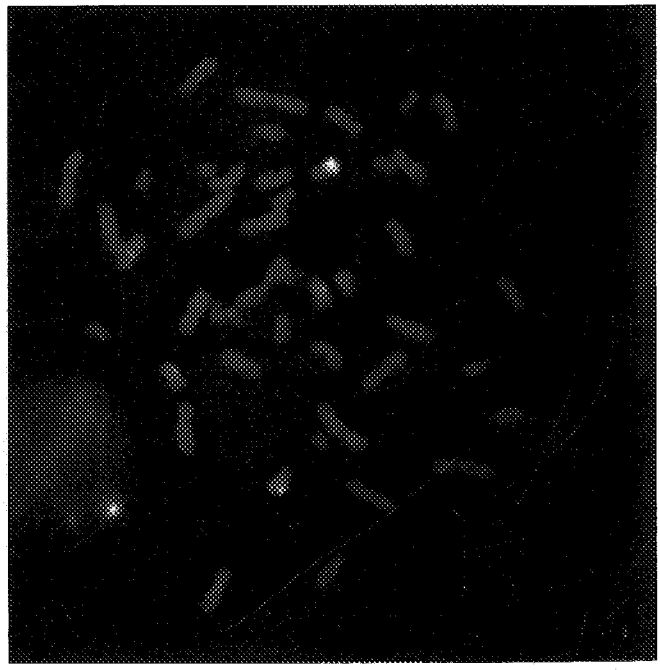

Figure 3. Raw picture of an EST990006 spread. Image before background reduction.

gov/genemap98/). This mapping result is consistent with the TSA-FISH mapping.

DISCUSSION

TSA-FISH is a relatively new protocol that allowed us to detect and to map probes as small as 855, 608 and 319 bp via increased signal amplification. However, as is often observed when mapping very small probes with conventional FISH, the smaller the probe, the lower the frequency of signals (symmetrical doublets) detected in metaphase spreads (5). With the 319-bp probe (Figure 2C) we were able to confirm the physical mapping of the gene, however the probe produced a low frequency of paired doublets, thus reflecting the limits of sensitivity of TSA-FISH for this gene sequence. However, with TSA-FISH, we found a much higher efficiency than expected, with 80% of the EST883227 metaphase spreads having at least one signal on each homologue.

Inherent with using small probes, many more spreads must be examined to have confidence in your mapping results. In addition, the technique is more sensitive to problems associated with high background. Therefore, with TSA-FISH, higher concentrations of probe are not recommended, as they would create greater background. These results have demonstrated the ability of TSA-FISH for mapping cDNAs using PCR-labeled probes. We also mapped for the first time, the human gene EST990006, a new human ABC gene, to 17q25.

The demonstrated sensitivity of TSA-FISH suggests this method might be applicable for the detection of other small sequence specific probes such as PCR fragments, microsatellites and sequence tag sites (STSs). As is true for conventional FISH mapping methods, several components are critical to insure success of the TSA-FISH protocol. The concentration of the DNA and the size of the probe should be determined before hybridization by running the PCR product on a gel and reading the OD_{260}. Regarding the concentration of the DNA, an average of 500 ng gives a good signal upon hybridization. Slides with metaphase spreads used for TSA-FISH must be clean and free of cytoplasm to ensure strong hybridization, decreased background and to raise the signal to noise ratio. The time of pepsin treatment and the denaturation time will vary for each preparation of metaphase spreads and must be optimized to ensure proper hybridization.

ACKNOWLEDGMENTS

The authors are grateful for assistance and advice from Dr. Evelin Schröck, (National Cancer Institute, Bethesda, MD). This project has been funded in part with Federal funds from the National Cancer Institute, National Institutes of Health under Contract No. NO1-CO-56000. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US Government.

REFERENCES

1. **Allikmets, R., B. Gerrard, A. Hutchinson and M. Dean.** 1993. Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the Expressed Sequence Tags Database. Hum. Mol. Genet. 5:1649-1655.
2. **Ballard, S.G. and D.C. Ward.** 1993. Fluorescence *in situ* hybridization using digital imaging microscopy. J. Histochem. Cytochem. *41*:1755-1759.
3. **Banerjee, M., F. Wiener, J. Spira, M. Babonits, M.G. Nilsson, J. Sumegi and G. Klein.** 1985. Mapping of the *c-myc*, *pvt-1* and immunoglobulin kappa genes in relation to the mouse plasmacytoma-associated variant (6;15) translocation breakpoint. EMBO J. *4*:3183-3188.
4. **Bobrow, M.N. and G.J. Litt, inventor;** E.I. DuPont DeNemours, assignee; Method for detection or quantitation of an analyte dependent enzyme activation system. US Patent No. 5196306. 1993.
5. **Dupont, B.R., V. Mattern and J.L. McCombs.** 1997. The Tackle Box: the smallest FISH. Appl. Cytogenet. *23*:133.
6. **Eilers, M., D. Picard, K. Yamamoto and J.M. Bishop.** 1989. Chimeras between the Myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature *340*:66-68.
7. **Henriksson, M. and B. Lüscher.** 1996. Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv. Cancer. Res. *68*:109-182.
8. **Janz S., J. Müller, J. Shaughnessy and M. Potter.** 1993. Detection of recombinations between *c-myc* and immunoglobulin switch α in murine plasma cell tumors and preneoplastic lesions by polymerase chain reaction. Proc. Natl. Acad. Sci. USA *90*:7361-7365.
9. **Kerstens, H.M., P.J. Poddighe and A.G. Hanselaar.** 1995. A novel *in situ* hybridization signal amplification method based on the deposition of biotinylated tyramine. J. Histochem. Cytochem. *43*:347-352.
10. **Litt, G.J.** 1996. A high-sensitivity signal enhancement method. Am. Biotechnol. Lab. Manual 16.
11. **Macville, M., T. Veldman, H. Padilla-Nash, D. Wangsa, P. O'Brien, E. Schröck and T. Ried.** 1997. Spectral karyotyping, a 24-colour FISH technique for the identification of chromosomal aberrations. Histochem. Cell Biol. *108*:299-305.
12. **Montgomery, K.D., E.A. Keitges and J. Meyne.** 1997. Molecular cytogenetics: definitions, clinical aspects, and protocols, p. 557-590. *In* M.J. Barch, T. Knutsen and J. Spurbeck (Eds.), The AGT Cytogenetics Laboratory Manual (3rd ed.). Lippincott-Raven, Philadelphia.
13. **Raap, A.K., M.P. van de Corput, R.A. Vervenne, R.P. van Gijlswijk, H.J. Tanke and J. Wiegant.** 1995. Ultra-sensitive FISH using peroxidase-mediated deposition of biotin- or fluorochrome tyramides. Hum. Mol. Genet. *4*:529-534.
14. **Raap, A.K.** 1998. Advances in fluorescence *in situ* hybridization. Mutat. Res. *400*:287-298.
15. **Speel, E.J., P. Saremaslani, J. Roth, A.H. Hopman and P. Komminoth.** 1998. Improved mRNA *in situ* hybridization on formaldehyde-fixed and paraffin-embedded tissue using signal amplification with different haptenized tyramides. Histochem. Cell Biol. *110*:571-577.
16. **Trask, B.J.** 1991. Fluorescence *in situ* hybridization: application in cytogenetics and gene mapping. Trends Genet. *7*:147-154.
17. **Van de Corput, M.P., R.W. Dirks, R.P. Van Gijlswijk, F.M. van de Rijke and A.K. Raap.** 1998. Fluorescence *in situ* hybridization using horseradish peroxidase-labeled oligodeoxynucleotides and tyramide signal amplification for sensitive DNA and mRNA detection. Histochem. Cell Biol. *110*:431-437.
18. **Van Gijlswijk, R.P., J. Wiegant, A.K. Raap and H.J. Tanke.** 1996. Improved localization of fluorescent tyramides for fluorescence *in situ* hybridization using dextran sulphate and polyvinyl alcohol. J. Histochem. Cytochem. *44*:389-392.
19. **Van Tine, B.A., J.F. Knops, A. Butler, P. Deloukas, G.M. Shaw and P.H. King.** 1998. Localization of HuC (ELAVL3) to chromosome 19p13.2 by fluorescence *in situ* hybridization utilizing a novel tyramide labeling technique. Genomics *53*:296-299.

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